

Obesity-induced Brain Insulin Resistance Exacerbates TBI Outcome

Thesis to Graduate with Distinction in the School of Health and
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Lindsey Freeman

Abstract

Traumatic brain injury (TBI) hospitalizes 1.7 million Americans every year and puts patients at risk for many complications including brain swelling, stroke, and behavioral, emotional and cognitive deficits. A common consequence of TBI is increased energy demand in the brain following injury, a phenomenon that has been attributed to a need to counteract the physical damage of impact. Our data suggest 1) that this compensatory mechanism is impaired when multiple TBIs occur close together in time, and 2) that this effect is driven by brain insulin resistance. The purpose of this study is to examine whether systemic insulin resistance prior to TBI (i.e. a model of type 2 diabetes) would further exacerbate TBI outcome. We used a diet-induced obesity model to create obese (intended to be insulin resistant) and lean cohorts of mice. Mice from both groups were randomly assigned to either a sham-injury procedure or two mild traumatic brain injuries (mTBI) 24 hours apart. Behavioral tests were used to assess anxiety-like behavior, motor coordination, and spatial learning/memory. Analysis of the brains indicated insulin resistance in both injured animals and diet-induced obese animals. Both diet-induced obesity and injury were correlated with greater cognitive deficits. These data support insulin insensitivity as a potential causal mechanism driving cognitive impairment in TBI. If this causal relationship among TBI and insulin resistance and cognitive deficits is established, then this work could conceivably lead to the treatment of TBI patients with insulin sensitizing drugs in order to reduce the severity of deficits.

Introduction

Traumatic brain injury (TBI) has become one of the greatest health concerns in the United States, with 1.7 million Americans affected each year⁵. While reported deaths related to TBI have decreased over the past decade, the rates of reported TBI have not¹⁹. Traumatic brain injury results from a violent blow to the head and, depending on its severity, can cause brain swelling and increased intracranial pressure, physical damage to brain tissue, and altered consciousness. Studies have linked these side effects to complications such as seizures, cranial nerve damage, degenerative brain diseases, and, most interestingly, cognitive impairments⁴. Survivors of TBI are left with cognitive deficits most commonly being memory loss and trouble planning, organizing, problem solving, and making judgments. Over 5 million Americans today need help performing activities of daily living because of previous TBI²³. The leading causes for TBI in the United States include falls (40% nationally but 81% within the elderly population), blunt trauma (15%), motor vehicle accident (14%), and assaults (10%)¹⁹. Taking these into account, there is virtually no group of persons in the country that is unaffected by TBI.

Severity of TBI varies and can be difficult to treat due to many other factors that need to be taken into account such as the patient's age, his or her personal health history and overall wellness, and how well he or she takes care of himself or herself after the injury. Also, there is a lack of a clear, standardized system by which to classify the severity of cases, making optimal treatment more difficult⁵. Much of modern treatment for TBI incorporates allowing the patient to rest and giving the brain an opportunity to restore its normal function over time. However, a persistent concern in this area of research is how to best determine the appropriate time for patients to return to activity – being work, school, military service, sports, etc. Once one injury has been sustained, a second injury becomes

more likely²². These repetitive TBIs present the threat of long-term neurologic and functional deficits.

The brain relies on a constant, regular supply of glucose for energy because of the absence of major stocks of glycogen or triglycerides (which are easily accessible stores of energy in other parts of the body like muscle and fat, respectively). This being said, the brain utilizes roughly 60% of all glucose consumed by an entire individual at rest².

Abnormal glucose utilization patterns present in the brain following a TBI. In a recent study, our lab showed that there is a period of increased glucose metabolism that peaks around six days after TBI. Following this hypermetabolic state, the brain actually enters a state of decreased glucose utilization. It is hypothesized that the initial hypermetabolic state following injury is compensatory for the damage induced by impact and fulfills the increased energy demand of the affected area. However, if a second injury occurs within days of the first, then the brain becomes incapable of mounting this response⁴.

Consequently, mice that experienced two mTBIs close in time could not meet the increased energetic demands, and they experienced greater behavioral outcome deficits than mice that only experienced one mTBI²⁵.

Here, we hypothesize that two mTBIs close in time cause a metabolic crisis mediated by impaired insulin signaling. There are emerging data indicating a pattern of central insulin resistance in other neurodegenerative brain states (e.g. Alzheimer's Disease^{1,6,7}), similar to the peripheral insulin resistance observed in T2D. Our study examined the possibility of insulin resistance as the mechanism behind cognitive deficits that occur following traumatic brain injury. Substantial research indicates chronic inflammation as a major cause of blood vessel constriction and subsequent tissue damage that leads to cognitive

deficiencies in this population¹⁷; however, the role of central metabolic processes in TBI-induced cognitive deficits is not well known. The aims of this study therefore were to 1) ascertain the role of insulin resistance in TBI and 2) determine how a predisposition to insulin resistance affects severity of cognitive deficits following repeated mTBIs.

Methods and Materials

Diet-induced Obesity

In order to predispose mice to insulin resistance, we used a maternal obesity model. Beginning at 4 weeks of age, female C57bl/6 mice were randomly assigned to either a low-fat standard chow diet or a high-fat diet⁸. These females were kept on this diet for 6 weeks before being paired with a male for breeding. Females continued on their respective diet throughout pregnancy and lactation, and their pups, the study animals, received the same diet as their mother after being weaned at 3 weeks of age. This model predisposes pups to obesity and symptoms of diabetes, and as such, insulin resistance. Weight gain was monitored throughout the study.

Traumatic Brain Injury

Six-week-old pups (both high fat; HFD, and low fat diets; LFD) underwent TBI or control (sham) procedure. The TBI model is a stereotaxic surgery that impacts an exposed skull at 3 m/s at the left somatosensory cortex. Mice were secured into the frame, a central incision was made to expose the skull, and the Stereotaxic Impactor One (Leica) was lowered to make contact with the skull. Contact with the bone was verified by the completion of an electric circuit between the injury device and the animal, and the position was recorded.

The pneumatic impactor was retracted, moved to coordinates AP: -1.0mm, ML: -1.0mm, and then accelerated into the skull 1mm below the surface. Mice in the TBI group sustained two identical injuries 24 hours apart in order to induce the targeted degree of brain injury without causing many fatalities. The control (sham) procedure followed identical methods excluding the impact. A total of 8-10 mice were included in each group for behavioral analysis, and an additional 7-9 mice were included per group for the insulin sensitivity assay (LFD/TBI, LFD/SHAM, HFD/TBI, HFD/SHAM).

Insulin Sensitivity

In order to measure central insulin resistance, brains were collected from all four groups 48 hours after surgery. Fresh slices of brain tissue were collected throughout the forebrain and cultured with 10nM insulin made in artificial cerebrospinal fluid (aCSF) or aCSF for 10 minutes. Total protein was then collected from the tissue. Protein analysis was conducted using western blotting to measure activation of phosphorylated Akt (pAkt S473), a downstream marker of functioning insulin sensitivity that is associated with neuroprotection. Insulin sensitivity was measured as a ratio of pAkt to total Akt following insulin stimulation⁴. GAPDH served as a loading control. Insulin hormone binding to its receptor, insulin receptor (IR), causes autophosphorylation of the receptor and activation of downstream targets such as IRS and Akt. Coupled with the critical role of Akt in neuroprotection and glucose storage¹⁰, the expression of activated Akt provides an excellent readout of both IR sensitivity and potential for neuroprotection.

Behavioral Analysis

Behavior testing was conducted beginning 3 days after TBI/SHAM surgery.

Rotarod

The rotarod test was performed to evaluate motor skill and coordination. It consists of a spinning platform that the animals must balance on to avoid falling. The mice were trained on increasing speed levels over three consecutive days prior to injury or sham, and their time taken to fall off the wheel was recorded for each day. Three days following surgery, mice were tested on time taken to fall with within a maximum time of five minutes.

Barnes Maze

Spatial learning and cognitive function were tested using the Barnes maze. This maze is a large circular platform with 20 holes around the circumference. One hole contains an escape box that takes advantage of rodents' natural aversion to light. The learning phase consists of 3 training sessions per day for five consecutive days, during which time the escape box is placed under the same hole every trial. Following the training, the escape box was removed, and the maze was divided into four equally sized quadrants. Mice were placed onto the maze for 90 seconds, and time spent in each quadrant was measured.

Light/Dark Box

Mice were placed in a box containing one open bright side and one dark enclosed side. They were first placed on the light side and remained in the box for a single 5-minute recorded trial. Based on the premise that rodents prefer to hide in dark enclosures, a greater length of time spent in the light is indicative of a lower level of anxiety-like behavior. The time spent on either side of the box, exploratory behavior (rearings), and time to first cross over (latency to enter dark side) were recorded.

Tissue collection

Brain tissue was collected following behavioral testing (approximately 3 weeks after TBI/SHAM procedure). Briefly, mice were overdosed with Euthasol, and transcardially perfused with saline and 4% paraformaldehyde. Following an overnight post-fix and cryoprotection in 30% sucrose, brains were sliced at a thickness of 60 μ m.

Histology

Degenerating neurons and axons were detected by using the FD NeuroSilver Kit (FD NeuroTechnologies) per the manufacturer's protocol. This application has been used successfully in our lab to demonstrate TBI-induced neurodegeneration. This marker provides a sensitive measure for the detection of axonal degeneration following TBI. Damage was assessed on a 0-3 scale (absent, mild, moderate, severe). Tissue was collected 3 weeks following TBI/SHAM procedure.

Statistical Analysis

Western blotting results and behavioral data were analyzed via 2-way ANOVA with diet and injury as the two independent variables. Silver staining was analyzed using Kruskal-Wallis analysis, a non-parametric equivalent of the ANOVA. For all data, a p-value of less than 0.05 was considered significant.

Required Facilities and Equipment

Mice were maintained in the BRT vivarium for the duration of the study under protocol number 2012A00000031 (Principal Investigator Zachary Weil). All mice had *ad libitum* access to food and filtered tap water, and housed in 14:10 light-dark cycle. The diets used were obtained from Research Diets, Inc. The high fat diet is product D12492 (60% calories from fat). The low fat diet was the control and is product D12450J (10% calories from fat). TBI and control procedures were performed by a research scientist in the Weil lab in a

procedure room in the BRT vivarium. I conducted behavioral testing in the Department of Neuroscience behavioral core lab in the BRT.

Results

Diet-induced insulin resistance

The maternal obesity model resulted in a significant increase in body mass of HFD-fed offspring compared to LFD (Figure 1). In order to confirm that this model produced central insulin resistance, SHAM and TBI brains from both diets were collected and treated with insulin. Our results indicate that among SHAM mice, the LFD produced a significant increase in Akt activation in response to insulin ($F_{1,9} = 8.089$, $p < 0.05$), however, the HFD brains did not exhibit insulin-dependent Akt activation. Moreover, TBI reduced insulin-dependent Akt phosphorylation in both LFD and HFD mice (Figure 2).

Marked Physiological Difference Between Sham and TBI

Immediately following TBI/SHAM surgery, mice were assessed for time to awake (TTA), although the TTA was significantly higher in TBI mice ($F_{3,32} = 13.692$, $p = 0.001$), there was no effect of diet on TTA. Analysis of the silver stain revealed that mice that had undergone TBI exhibited a distinct increase in neuronal degeneration ($H_3 = 29.464$, $p < 0.001$), which did not differ by diet ($p > 0.05$). See Figure 3.

Motor skills were unaffected by TBI or diet

Throughout the three training days on the rotarod test of motor skill and coordination, no significant differences between any of the four groups in time taken to fall were observed ($p > 0.05$ for all comparisons). Similarly, on testing (3 days after TBI/SHAM) mice performed the same on rotarod performance regardless of surgery or

diet, indicating that motor skills were unaffected by our manipulations ($p > 0.05$ for all comparisons). See Figure 4.

HFD-induced obesity enhances anxiety-like behavior following TBI

Latency to cross indicated that injured HFD mice took significantly less time to cross over to the dark side compared to HFD shams ($F_{1,15} = 4.688$, $p = 0.05$), indicating that the HFD resulted in increased anxiety-like behavior following TBI. Indeed, there was no significant difference in latency to enter the dark box within the LFD cohort ($p < 0.05$). Injured mice in the HFD cohort also spent significantly less time on the light side of the box compared to the non-injured mice in the HFD cohort ($F_{1,14} = 5.253$, $p < 0.05$). Again, no significant difference existed for this measure within the LFD cohort ($p > 0.05$). See Figure 5.

HFD significantly impaired memory on the Barnes Maze

Over the five training days, no significant difference in latency to learn the task was observed among any of the four groups ($p > 0.05$ for all comparisons). However, on the “probe” day, HFD mice spent significantly less time in the target quadrant than LFD mice ($F_{1,32} = 4.317$, $p < 0.05$). See Figure 6.

Discussion

The purpose of this study was to examine insulin resistance as a potential mechanism underlying TBI-induced cognitive deficits. Our results indicate that repeated TBIs produce marked insulin resistance in the brain. Using a maternal diet-induced model of obesity, we were able to render offspring insulin resistant. A behavioral analysis of HFD and LFD mice following TBI revealed greater anxiety and memory deficits in HFD/TBI

mice. Taken together, these data indicate a possible link between central insulin resistance and functional outcomes following TBI.

Glucose, which is a product of broken-down foods, stimulates the release of insulin by the beta cells of the pancreas¹⁸. Typically, insulin crosses the blood-brain barrier and binds to insulin receptors in the central nervous system (CNS), leading to the phosphorylation of insulin receptor substrate (IRS) proteins. Insulin's binding to its receptor and appropriate downstream phosphorylating effects act as a key to unlock glucose's pathway into cells throughout the body^{10,11}. Studies have established a direct link between neuronal insulin receptor signaling and energy regulation^{9,18}.

Insulin resistance is a condition in which insulin receptors fail to respond to the hormone itself. An important mechanism by which insulin resistance is believed to occur involves altered insulin receptor substrate protein (IRS-1) signaling. Disease states (i.e. brain injury or obesity) affect insulin receptor signaling via a mechanism that inhibits phosphorylation (and thus causes *deactivation*) of IRS-1, a protein that typically is responsible for the downstream signaling and activity of insulin receptors. Similarly, TBI-induced neuroinflammation results in the production of cytokines and chemokines, both of which also inactivate IRS-1¹⁰. IRS-1 homeostatically participates in a negative feedback loop where the abundance of downstream products in the insulin receptor signaling pathway in turn down-regulates IRS-1 activity. When this loop is up-regulated, as it is in both insulin resistance and TBI, insulin receptor insensitivity occurs, leading to hyperglycemia in the blood. Moreover, activation of Akt, a downstream target of IRS-1 and a critical neuroprotectant, via this pathway is inhibited following IRS-1 deactivation. This

reduction in insulin-dependent Akt signaling removes a critical anti-inflammatory response, thus impairing the capacity for tissue repair after TBI^{14,16}.

This pattern of insulin insensitivity is not unlike that which is observed peripherally in Type 2 diabetes (T2D). In fact, insulin resistance is a principal component of the pathophysiology of type 2 diabetes. A strong association has been observed between T2D and increased risk for neurodegenerative disease^{1,18,24}. Importantly, the insulin insensitivity observed in our study is similar to what has been found in Alzheimer's brains^{6,7}. Indeed, some of the cognitive deficits associated with Alzheimer's disease are linked to impaired brain metabolism. A substantial body of work confirms that insulin resistance is linked to declining cognitive function, hippocampal plasticity, and Alzheimer's associated neuropathology^{7,9,21}. Our data suggest that the same mechanism may be responsible for cognitive deficits following TBI.

Anxiety and depression are very common after TBI¹³. The entire spectrum of anxiety disorders has been reported to some extent following mild TBI, and nearly a quarter of TBI patients are thought to suffer from an anxiety disorder¹⁵. Our results show increased levels of anxiety-like behavior in insulin-resistant (HFD) animals following TBI. These are similar to what has been published previously^{3,20}. Evidence suggests that anxiety and other psychiatric disorders may exert a negative influence of the course of recovery for TBI patients¹⁵. This may be due to poorer adherence to treatment, a poorer overall quality of life, an increased risk of developing a substance abuse disorder, or many more social and contextual consequences of life with anxiety or depression. A significant increase in anxiety levels was only observed in HFD animals in our study, which supports the hypothesis of insulin resistance as a potential mechanism for TBI-induced anxiety. It's important to note

that the extent of neuronal damage was similar between diets (Figure 3), so the increased anxiety cannot be attributed to increased damage with respect to any measure we performed, but rather seems to directly be a result of diet-induced obesity and insulin resistance.

In conclusion, our study confirms the presence of central insulin insensitivity following TBI, persisting for at least 48 hours after injury. Moreover, rendering mice insulin resistant prior to TBI resulted in greater cognitive deficits. We are currently conducting further experiments to determine the time course of recovery of insulin sensitivity following TBI. Identifying the course of insulin insensitivity following TBI may become pertinent to recognizing when it is safe for a TBI patient to return to activity, particularly given the fact that a single TBI puts an individual at a much greater risk for sustaining additional, often debilitating¹², TBIs. Given that the recovery of cognitive function correlates strongly with the return of normal glucose metabolism in both humans and animals, future development of a biomarker (i.e. insulin sensitivity) that can be used to determine when it is safest for a TBI patient to return to his/her regular activities is a goal of the clinical future.

The presence of insulin resistance in injured brains provides greater understanding of the damage that is incurred by chronic TBIs. Furthermore, the relationship between Type 2 Diabetes, of which insulin resistance is the quintessential symptom, and neurodegenerative disease furthers the hypothesis that insulin resistance may causally related to impaired cognitive and behavioral performance in patients suffering from TBI, T2D, or neurodegenerative disease alike. Many pharmacological treatments have been tested as potential therapies to reduce or deter neurological damage caused by TBI, but

none have proven broadly effective thus far. Should future findings continue to suggest insulin resistance as a causal mechanism behind these deficits, an investigation of a treatment approach targeting insulin sensitivity to help the brain meet the high energy demands of repair and recovery following TBI is warranted. Early clinical trials of such treatments are underway for Alzheimer's patients²¹, and preliminary data provide direction for future studies.

Figures

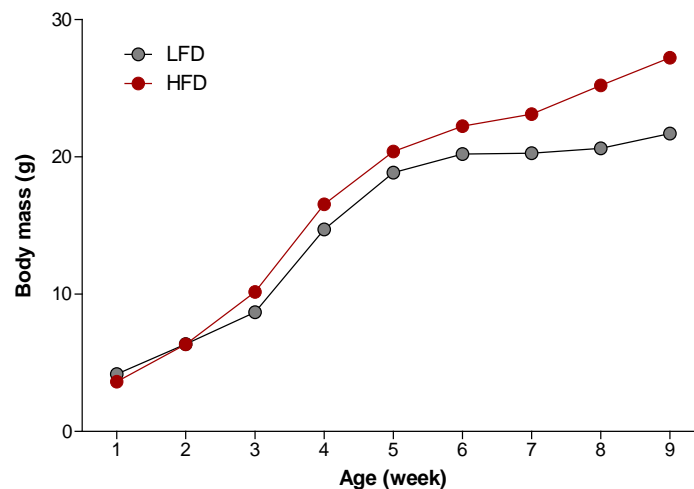


Figure 1: Body mass of mice throughout study

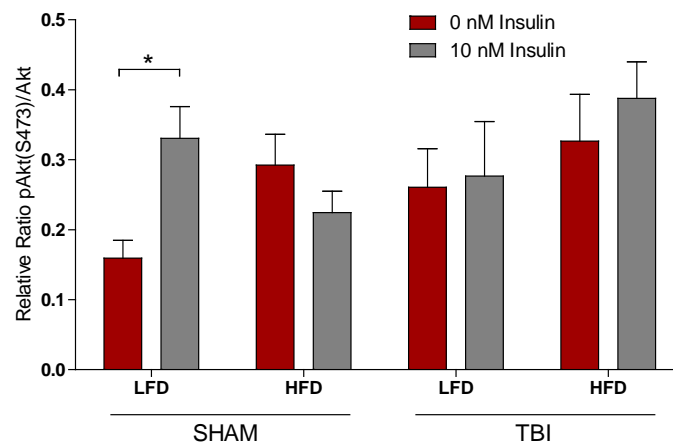


Figure 2: Brain insulin resistance following HFD and TBI. Insulin receptor sensitivity was measured via acute slice culture preparation following Sham or TBI injury across both diets. Brain slices were collected 48 hours following injury and cultured with insulin (10nM) or aCSF control for 10 minutes. Insulin resistance was measured via western blotting for phosphorylated Akt (pAkt S473), a target site downstream of the insulin receptor that is associated with neuroprotection. The graph shows an increase in Akt phosphorylation in LFD but not HFD sham mice, indicating a loss of insulin sensitivity in the HFD group. Repeated TBI led to marked insulin resistance in both LFD and HFD mice. Data are shown as mean \pm SEM. An asterisk (*) indicates $p < 0.05$.

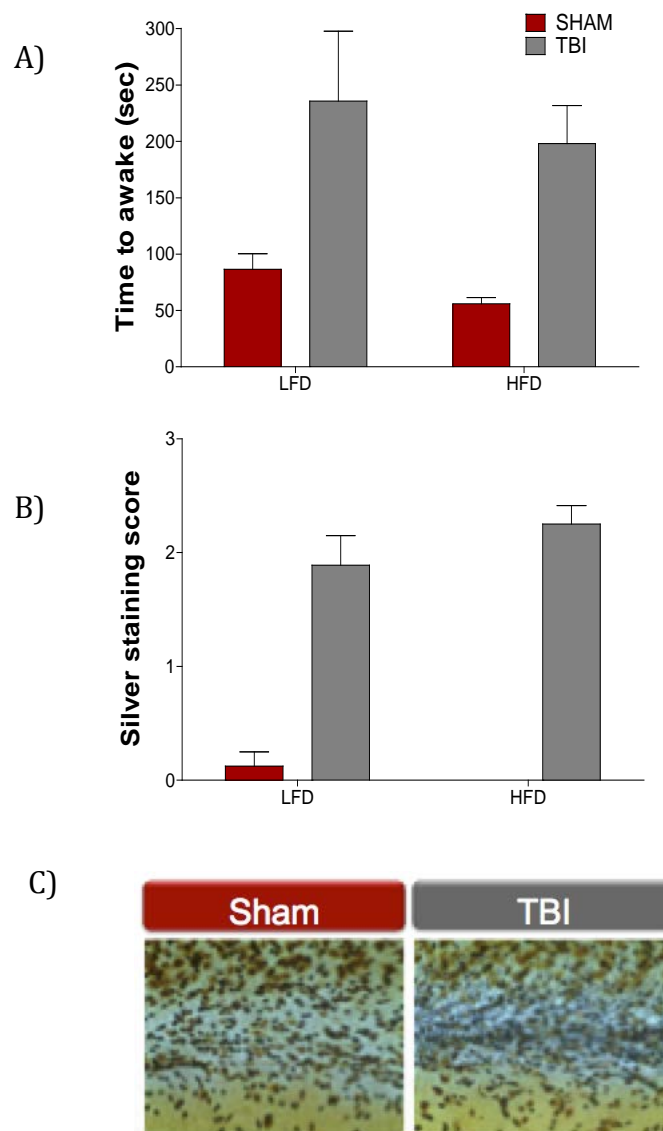


Figure 3: TBI severity and axonal damage. (A) TBI significantly increases the time to awake (TTA) following anesthesia removal. TTA was not affected by the diet. (B) The severity of brain damage was assessed by silver staining which allows for visualization of axon degeneration. Mice undergoing TBI exhibited a distinct increase in axon damage, which did not differ by diet. (C) Shown are representative images of the corpus callosum following Sham and TBI injury. Data are shown as mean \pm SEM.

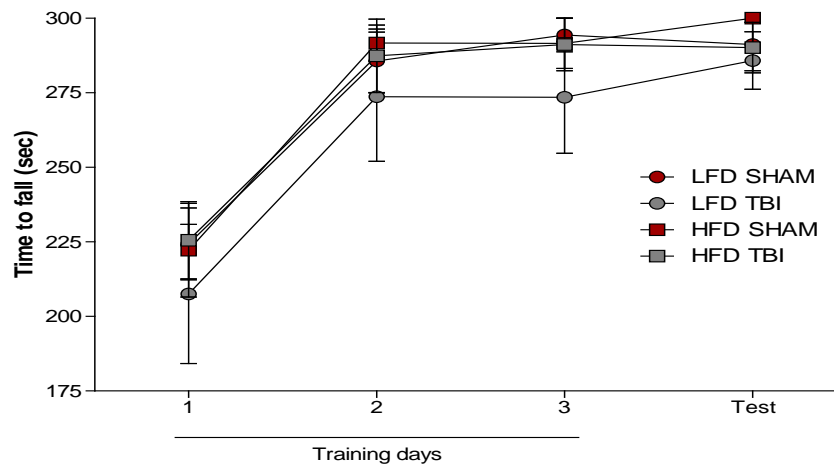


Figure 4: Motor skills were unaffected by TBI or diet. The rotarod test measures motor skill and coordination. Training was conducted over three days prior to Sham/TBI, and a final test was conducted 3 days following injury. Performance was not significantly impaired for any group across diet or injury. Data are shown as mean \pm SEM.

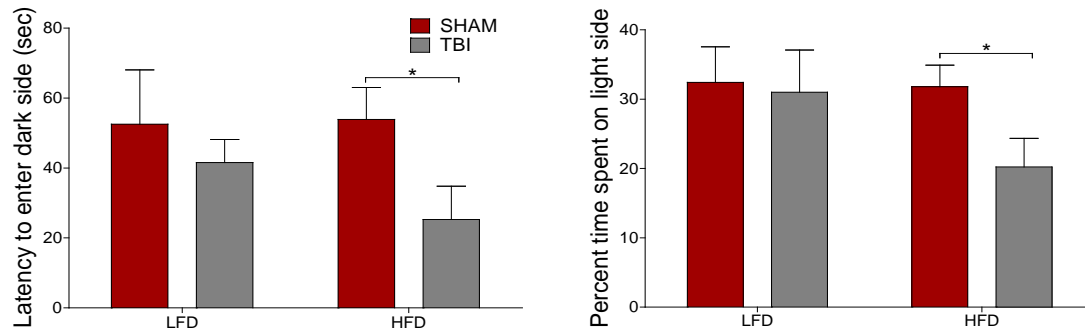


Figure 5: HFD-induced obesity enhances anxiety following TBI. The light/dark box test assesses anxiety-like behavior through measures of latency to enter the dark side of the box (left) and total time spent on the light side of the box (right), exploiting rodents' natural aversion to light. A significant difference was observed for both of these measures between injured and non-injured mice in the HFD cohort, while no significant difference existed for either in the LFD cohort. Data are shown as mean \pm SEM. An asterisk (*) indicates $p < 0.05$.

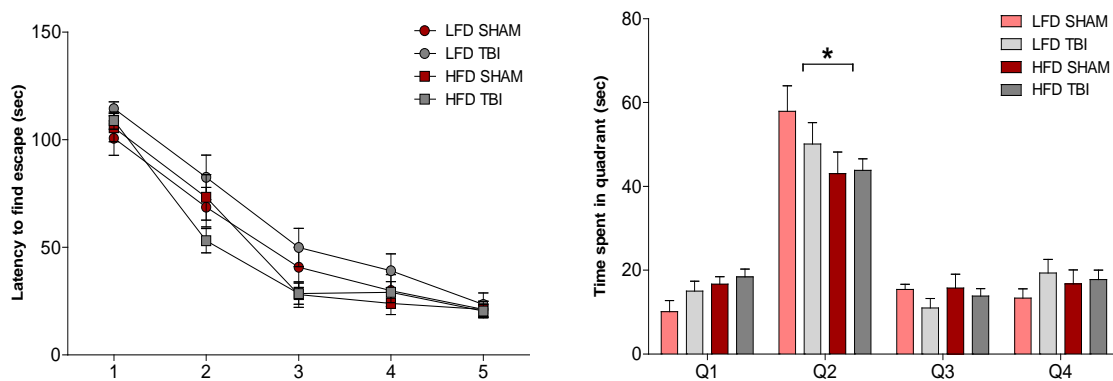


Figure 6: HFD significantly impaired memory on the Barnes Maze. Performance on the Barnes maze test of spatial learning and memory was measured 2 weeks following Sham/TBI. There was no significant overall difference in latency to learn the task over a 5-

day period (left). On the “probe” day, HFD mice spent significantly less time in the target quadrant, indicating memory impairment relative to LFD mice (right). Data are shown as mean \pm SEM. An asterisk (*) indicates $p < 0.05$.

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